A METHOD FOR THE QUANTITATIVE ESTIMATION OF CYTOPLASMIC STRUCTURES

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ABSTRACT

A sampling procedure and calculations are described by which electron micrographs of cytoplasmic structures may be quantitatively analyzed. The relative areas occupied by formed bodies and by the "membrane space," the remainder of the cytoplasm, are evaluated. A method for making a measurement of the quantity of endoplasmic reticulum or other membrane profiles is described. The technic and results are illustrated with normal rat liver cells.

INTRODUCTION

Electron microscopic studies of biological objects, especially the thin section of cells, have been principally concerned with qualitative descriptions of structures. For comparing cells in which only relatively small quantitative differences in composition are found, it is desirable to have a systematic technic for the estimation of structures. Chalkley *et al.* (1) have described a method based on a statistical sampling of tissue sections for determining the volume-surface ratio in morphologic components. A rigorous mathematical treatment has been given of the analogous technic of lineal analysis employed in metallurgical studies (8). Quantitative analysis of the subcellular architecture revealed by electron microscopy is a logical step in the investigation of the normal functioning of cells and their pathological alterations. A simple method for the evaluation of the relative areas occupied by formed structures, *e.g.* nuclei, mitochondria, and inclusion bodies, is described. Furthermore a technic is proposed for quantitating the endoplasmic reticulum by measuring the length of its irregular profile appearing in thin sections. Although normal rat liver cells have been used for illustration, the technic is clearly applicable to diverse tissues.

METHODS

Small pieces of fresh liver were removed from three normal, 140 to 160 gram, female Fisher rats and were fixed for 2 hours in cold pH 7.4 buffered 0.6 per cent potassium permanganate solution (6). The tissues were embedded in the water-miscible resin fraction Aquon as described by Gibbons (3). Thin sections were cut with a Porter-Blum ultramicrotome using a diamond knife. An RCA EMU-2A electron microscope was used for the observation and photographing of selected cells.

In order to avoid prejudice in selecting cells whose cytoplasmic composition was to be measured, the sampling was done visually by gross criteria in the electron microscope at a magnification of less than 2000. Under these conditions fine structural details were not discernible. To maintain uniformity in selection, only those cells were micrographed in which the nucleus was visible, indicating that the section passed approximately through the center of the cell.

Measurements of cells were made on prints enlarged to 11 × 14 inches at magnifications of 15,000 to 30,000. A grid consisting of 13 parallel lines spaced 25.4 mm apart was laid over the electron micrograph as illustrated in Fig. 1. The grid was constructed from a rectangular frame with strands of no. 28 gauge steel wire stretched across the opening.

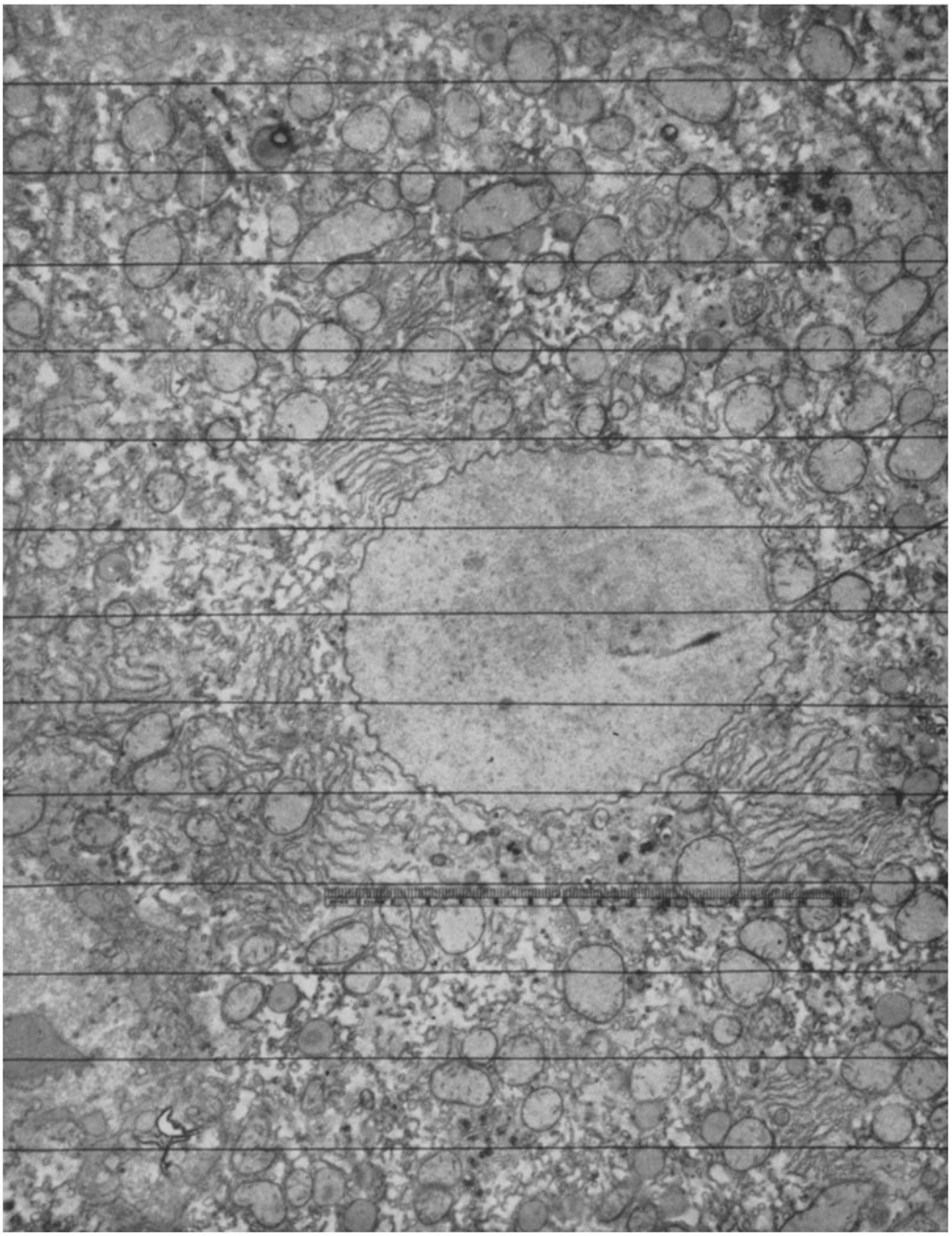

FIGURE 1

Electron micrograph of a normal rat liver cell as it appears in the counting frame with the grid of 13 parallel lines superimposed, reproduced at one-half actual size. The measurements on this micrograph are shown in Table I.

The total length of these sampling lines actually lying on the picture is 13 times the width of the photograph exclusive of margins. Distances along this line were measured by placing the millimeter scale of a ruler close to the wire line (see Fig. 1). For the examples given here the pictures were 280 mm wide and the total line was therefore 3640 mm.

The area composition of a micrograph was estimated from the fraction of the total sampling line that passed over such defined figures as the nucleus, mitochondria, lipid bodies, microbodies, and intercellular spaces. Each group of structures, *e.g.* mitochondria, was measured individually, and the number of millimeters of the line traversing those cross-sections was recorded. Measurements on each of the parallel lines were tabulated separately as shown in Table I, an illustrative data sheet for the micrograph seen in Fig. 1. The eighth column of Table I shows the number of millimeters of the line, calculated by difference, which overlies areas of the cytoplasm not included in the afore-mentioned formed structures. This figure represents the remainder of the cytoplasm, in which the principal structures are the irregularly shaped and dispersed membranes of the endoplasmic reticulum and which is here termed the "membrane space."

To obtain a quantitative measurement of the membrane profiles in the membrane space, a counting method was used. The figures in the last column of Table I, called "crossings," are counts of the number of intersections between the wire line and the profiles or edges of the membranes of endoplasmic reticulum in the electron micrograph. Tangential contacts were also counted, and obliquely sectioned membranes were included if they could be identified clearly.

CALCULATIONS AND RESULTS

The data for each cell measured were recorded as shown in Table I. Then the sum of each column was determined. At this point the area of the

TABLE I

*Data Sheet for Quantitative Measurement of Cell Structures**

Subject: Normal rat liver cell; $KMnO_4$, Aquon.
Plate: 2164 *e*.
Print magnification (M): 16,500.

Row	Width	Intercellular space	Nucleus	Mitochondria	Microbodies	Lipid bodies	Membrane space	Crossings (C)
1	280	0	0	53	0	0	227	42
2	280	12	0	64	6	0	198	53
3	280	0	0	109	12	0	159	51
4	280	0	0	112	9	0	159	68
5	280	0	0	45	0	0	235	52
6	280	0	108	6	11	0	155	38
7	280	0	125	44	0	0	111	38
8	280	0	111	0	12	0	157	45
9	280	0	52	52	16	0	160	48
10	280	17	0	49	0	0	214	63
11	280	39	0	43	10	0	188	71
12	280	52	0	68	6	0	152	50
13	280	60	0	32	9	0	179	56
Totals	3640	180	396	677	91	0	2294	675
% total area		4.9	10.9	18.6	2.5	0	63.0	
Total cytoplasm (L)		3062						
% cytoplasmic area				22.1	3.0	0	74.9	

$$\text{Membrane profile concentration} = \frac{\pi CM}{2000L} = \frac{(3.14)(675)(16{,}500)}{(2000)(3062)} = 5.71.$$

* See text for explanation of the figures and calculations.

whole electron micrograph was analyzed by multiplying the total for each component by the fraction 100/3640. This gives the percentage area occupied by each component (see Table I). If only the cytoplasm is to be studied, its total area is represented by the combined totals of mitochondria, microbodies, lipid bodies, and membrane space, designated by L. The percentage of cytoplasmic area occupied by each of these components was calculated as above by using $100/L$.

TABLE II

Multiple Measurements of Cells with Varying Grid Position

Cell	Position*	% area of cytoplasmic cross-section: Mitochondria	Microbodies	Lipid bodies	Membrane space	Total crossings	Membrane profile concentration
2081 *c*	Normal	15.7	1.6	0.3	82.4	696	5.29
	1/2″	15.1	0.3	0.0	84.6	568	4.66
	−45°	17.2	0.8	0.2	81.8	553	4.21
	+45°	17.4	0.6	0.4	81.6	593	4.54
	90°	15.7	1.4	0.0	82.9	583	4.36
Average (S.E.)		16.2 (0.9)	0.9 (0.5)	0.2 (0.2)	82.7 (1.1)		4.61 (0.37)
2097 *c*	Normal	17.9	1.7	0.5	79.9	610	4.68
	1/2″	16.7	1.3	0.7	81.3	729	5.59
	−45°	14.1	0.7	0.7	84.5	764	5.47
	+45°	16.5	1.1	0.1	82.5	661	4.76
	90°	17.2	1.5	0.1	81.2	804	5.76
Average (S.E.)		16.5 (1.3)	1.3 (0.3)	0.4 (0.3)	81.9 (1.6)		5.25 (0.44)
2164 *e*	Normal	22.1	3.0	0.0	74.9	675	5.71
	1/2″	17.2	2.4	0.0	78.3	730	6.27
	−45°	19.3	3.3	0.0	78.6	758	6.30
	+45°	19.4	2.0	0.0	77.4	642	5.23
	90°	19.3	2.4	0.0	80.4	799	6.80
Average (S.E.)		19.5 (1.6)	2.6 (0.5)	0.0 (0.0)	77.9 (1.8)		6.06 (0.54)

* Five positions of the grid of parallel lines were used: Normal, as illustrated in Fig. 1; 1/2″, similar to the normal position but with grid displaced 1/2 inch downward; −45°, grid rotated 45° counterclockwise; +45°, grid rotated 45° clockwise; 90°, grid rotated 90°.

The cross-sections of the membranes of endoplasmic reticulum appear as tortuous lines in electron micrographs. A measure of the length of these lines, or profiles, in the micrograph can be calculated from the frequency with which they intersect the sampling line. Expressed per unit area of the cytoplasm, this figure shows the concentration of endoplasmic reticulum in the cytoplasm. This calculation, which is shown at the bottom of Table I, is based on the recognition that the "crossings" count is related to the classical needle problem of Buffon (4). In this problem a needle of length d is tossed at random orientations on a field of parallel lines having a uniform spacing D (greater than d) between the lines. In a large number of trials, n, the number of tosses, C, in which the needle lies across a line is given by

$$C = \frac{2nd}{\pi D}. \quad (1)$$

Rearranging this equation to the form

$$nd = \frac{\pi CD}{2}, \quad (2)$$

it is seen that the variables on the right hand side correspond to values available in the present data: D equals 25.4 mm and C is the number of cross-

ings. The left hand side, *nd*, is equivalent to the sum of all the needle lengths cast. Since the elementary configurations of the endoplasmic reticulum are generally smaller than the grid spacing used, these profiles may be thought of as being composed of a large number, *n*, of randomly oriented segments with the small length *d*. Thus, equation (2) is an estimate of the total length (in millimeters) of the cross-section of endoplasmic reticulum appearing in the sampled area. Dividing this figure by *DL* (*L* being the number of millimeters of sampling line traversing the cytoplasmic area), the area sampled, gives the millimeters of profile per square millimeter of cytoplasm in the micrograph:

$$\pi C/2L.$$

Finally, multiplying by $M/1000$ (M equals the total magnification in the photographic print) reduces the expression to the unmagnified dimensions of the cell and defines the "membrane profile concentration":

$$\text{"Membrane profile concentration"} = \frac{\pi CM}{2000L}$$

= average number of microns of endoplasmic reticulum profile per square micron of cytoplasm in the unmagnified cell cross-section.

The magnification of micrographs used in these analyses must be great enough to permit accurate counts and measurements. Too much magnification, however, results in an inadequate sampling of the cytoplasm. Since the statistics of the number of "crossings" is similar to that of cell counts obtained with a hemocytometer, the standard error is given by the square root of the number of crossings counted (2). Thus, for a count of 400 the standard error is 20, or 5 per cent of the total. This level of accuracy, a minimum of 400 crossings counted in each micrograph, has been maintained in all cells measured.

In order to obtain a larger sample from a particular cell, the grid may be applied to the micrograph in different positions and orientations and the measurements repeated. Table II presents the results of multiple measurements on three micrographs. It is seen that the figures for the distribution of cytoplasmic area are highly reproducible for each position of the grid. On the other hand, the variation in the measurement of membrane profile concentration among repeated samples on the same micrograph is somewhat larger than would be expected. Whereas a standard error of 3.5 to 4.3 per cent may be estimated from the range of crossings counted (804 to 553), the actual standard errors calculated from the data in Table II are 8 to 9 per cent of the average membrane profile concentrations. The additional variation probably originates from the coincidental align-

TABLE III

Cytoplasmic Composition of Normal Rat Liver Cells

Cell	% area of cytoplasmic cross-section				Membrane profile concentration*
	Mitochondria	Microbodies	Lipid bodies	Membrane space	
2080 *a*	15.1	0.4	0.0	84.5	5.15
2081 *c*	16.2	0.9	0.2	82.7	4.61
2082 *e*	21.5	1.8	0.8	75.9	4.04
2083 *e*	20.3	0.9	0.5	78.3	4.53
2097 *c*	16.5	1.3	0.4	81.9	5.25
2162 *e*	19.9	0.4	0.0	79.7	6.08
2163 *e*	20.8	1.2	2.8	75.2	5.42
2164 *e*	19.5	2.6	0.0	77.9	6.06
2211 *d*	19.6	1.6	0.9	77.9	5.56
2213 *b*	18.1	2.1	1.6	78.2	4.29
2214 *d*	15.3	2.0	0.0	82.7	5.69
Average(S.E.)	18.4 (2.2)	1.4 (0.7)	0.7 (0.8)	79.5 (2.9)	5.15 (0.67)

* The membrane profile concentration is defined as the average number of microns of endoplasmic reticulum profile per square micron of cytoplasm in the unmagnified cell cross-section.

ment of the grid lines with respect to parallel arrays of cytoplasmic membranes. This result emphasizes the desirability of combining repeated measurements at different positions of the sampling lines to eliminate any orientation effect.

Table III shows the results of analyses of the cytoplasmic composition of randomly selected normal rat liver cells. The average values and their standard errors are given below each column. Measurements of this kind reveal the variations present in similar cells and can form the basis for subsequent comparisons.

DISCUSSION

The method described here leads to a quantitative evaluation of biological structures seen in electron micrographs independent of their shape and distribution. In cross-sections of cells the relative area occupied by formed bodies, such as nuclei, mitochondria, lipid bodies, and other inclusions, can be measured. The remainder of the cytoplasmic area has been termed the "membrane space" because it represents the region whose principal component is the membranous endoplasmic reticulum. The concept of membrane space is significant in relation to the cell fractions produced from homogenates, inasmuch as microsomes, ribosomes, and soluble cell components are all derived from this region. By the technic of counting the intersections between the edge, or profile, of the endoplasmic reticulum and the sampling line superimposed on the micrograph, the length of this edge can be estimated. The length of membrane edge per unit area of cytoplasm (unmagnified) is expressed by the "membrane profile concentration," a measure of the amount of membranous organization in the cytoplasm.

These quantitative measurements from two-dimensional cross-sections can be extended to the real three-dimensional structure of the cell. The fraction of cytoplasmic volume occupied by formed bodies and membrane space is numerically identical with the area fractions already described. Thus, from Table III, 18.4 per cent of the cytoplasmic volume of the average cell of the normal rat liver is composed of mitochondria; 1.4 per cent, microbodies; etc. Smith and Guttman (8) showed that "the average number of intercepts per unit length of a random line drawn through a three-dimensional structure is exactly half the true ratio of surface to volume." This result may be used to calculate the "membrane surface concentration" in cells. The membrane surface concentration is defined as the number of square microns of endoplasmic reticulum per cubic micron of cytoplasm. In the present notation this value is readily shown to be

$$\frac{C}{L} \times \frac{M}{1000} \times 2$$

or, more simply, $4/\pi$ times the membrane profile concentration.

From Table III and the foregoing definition the membrane surface concentration in the normal rat liver cell is calculated to be 6.56 square microns of endoplasmic reticulum membrane per cubic micron of cytoplasm. The actual area of structural interface is twice this value, since the membrane has two sides, one facing the cisternal cavities and one facing the other cytoplasmic constituents (7). This computation is valid for comparative studies but it should be considered only a minimal estimate of the surface present in the cell. Williams and Kallman (9) have shown that membranes cut obliquely in thin sections are not visible unless oriented nearly perpendicular to the plane of the section. Thus, the cytoplasmic membranes are at most only partially visualized by electron microscopy. The calculation of three-dimensional properties is strictly correct only when the areas to be measured are selected truly at random. However, the sampling method used here, the deliberate choice of central sections, should not introduce significant error unless the peripheral cytoplasm is distinctly different in structure from the more central regions.

With the methods described here a quantitative analysis of cytoplasmic ultrastructure can be used to complement parallel metabolic or biochemical studies. Numerical evaluation of structures heretofore qualitatively described in electron micrographs enables their study by statistical technics. In this way, for example, the electron microscope may be utilized more effectively to test and to demonstrate the relatedness of cellular structures and functions.

The substance of this article was presented at the 19th Annual Meeting of the Electron Microscope Society of America in Pittsburgh, Pennsylvania, August 23, 1961 (5). This study was supported in part by United States Public Health Service Re-

search Grant C-4307 and in part by an Institutional Grant from the United Foundation of Greater Detroit allocated through the Michigan Cancer Foundation.

The technical assistance of Miss Barbara Guinness and Mr. Frederick F. Schaub, Jr., is gratefully acknowledged.

Received for publication, November 30, 1961.

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